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TITLE: Role of NF-Kappa B Signaling in X-Box Binding Protein 1 (XBP1)-Mediated Antiestrogen Resistance in Breast Cancer

PRINCIPAL INVESTIGATOR: Dr. Rong Hu

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057-0001

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14. ABSTRACT
Most breast cancer patients who undertake antiestrogen therapy eventually suffers from antiestrogen resistance. Understanding its molecular mechanism is essential for identifying potential targets to overcome antiestrogen resistance. XBP1-S, an important regulator of the unfolded protein response (UPR), is found highly expressed in antiestrogen resistant breast cancer cells and tissues. XBP1-S is believed to function as an important antiestrogen resistance mediator as overexpression of XBP1-S is sufficient to drive resistancy to antiestrogens in MCF7 cells. In this study, we aim to investigate the mechanism of XBP1-mediated antiestorgen resistance, specifically the involvement of NFkappaB signaling. We found that XBP1 regulates NFkappaB signaling in an ERalpha signaling dependent mechanism. We have demonstrated that both XBP1(U) and XBP1(S) can interact and activate ERalpha but not ERbeta. We have also used both ERalpha positive and negative breast cancer cell lines to show that ERalpha signaling is essential for XBP1 activated NFkappaB signaling. In addition, we have examined the role of XBP1(U) and XBP1(S) in tumor development in vivo. We have injected nude mices with MCF7 cells that overexpress XBP1 and lacZ control cells. As expected, we observed enhanced growth in XBP1 overexpressed cells. However, the tumor growth of XBP1(U) overexpressed cells was significantly faster than XBP1(S) cells. We are currently examining the potential mechanism for this observation.

15. SUBJECT TERMS
None provided.

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I. Introduction:

Antiestrogen is the most widely used effective treatment to ER+ breast cancer patientsnowadays. However, antiestrogen resistance has long beenthe major hurdle for endocrine therapy (1). As the UPR major component,XBP1 was shown to be up-regulated in antiestrogen resistant breast cancer cells and tumors (2-4). XBP1 overexpression is sufficient to promote resistance to antiestrogen in breast cancer (2). However, the underlying mechanisms remain to be clarified. NFkappaB signaling is known to be up-regulated in antiestrogen resistant cells and inhibition of NFkappaB re-sensitizes cells to antiestrogen (5). Our hypothesis is that NFkappaB is downstream of XBP1 signaling and mediates the antiestrogen resistance signaling.

II. Research Accomplishment Body:

Aim 1: Determine whether the XBP1-mediated antiestrogen resistance is mediated through NFkappaB signaling.

The first year's research, we established a link between XBP1 and NFkappaB signaling. We found that NFkappaB signaling is required for XBP1 mediated antiestrogen resistance. Inhibition of XBP1 in antiestrogen resistant cell lines will inhibit NFkappaB signaling, and overexpression of XBP1 in sensitive MCF7 cells will also enhance NFkappaB signaling. We found that XBP1 regulates p65/RelA expression level at the mRNA level. More interestingly, we found that XBP1(U), which was the unspliced and supposingly inactive form of XBP1, can also regulates NFkappaB activity. Our last year's results suggested ER-alpha as a potential mechanism for this regulation. Therefore, based on our last year's results, we furthered our research this year to further investigate the interaction between XBP1 and NFkappaB signaling, especially the role of ER alpha.

To understand how XBP1(S) and XBP1(U) regulates ER-alpha, we have transfected various constructs of XBP1 (overexpress XBP1(S) and/or XBP1(U)) into MCF7 cells and examined the level of ER-alpha signaling with ERE-luciferase activity**(Figure 1A)**. We have found that overexpression of both XBP1(S) and XBP1(U) are able to up-regulate ERE-luc activity. This result is consistent to what we have observed with NFkappaB-luc, suggesting a positive link between ER-alpha signaling and NFkappaB signaling in these breast cancer cells. ER-alpha has previously been shown to be able to directly bind to bothXBP1(U) and XBP1(S), and modulates its transcriptional activity. To determine whether this interaction exists in our system, we have performed immunoprecipitation experiments with cells overexpress both ER-alpha and XBP1(U) or XBP1(S) **(Figure 1B)**. We found that both XBP1(U) and XBP1(S) can be co-immunoprecipitated with ER-alpha, confirming the interaction between both forms of XBP1 with ER-alpha. We have also tested their binding to ER-beta and observed no interaction, suggesting the interaction is specific to ER-alpha.

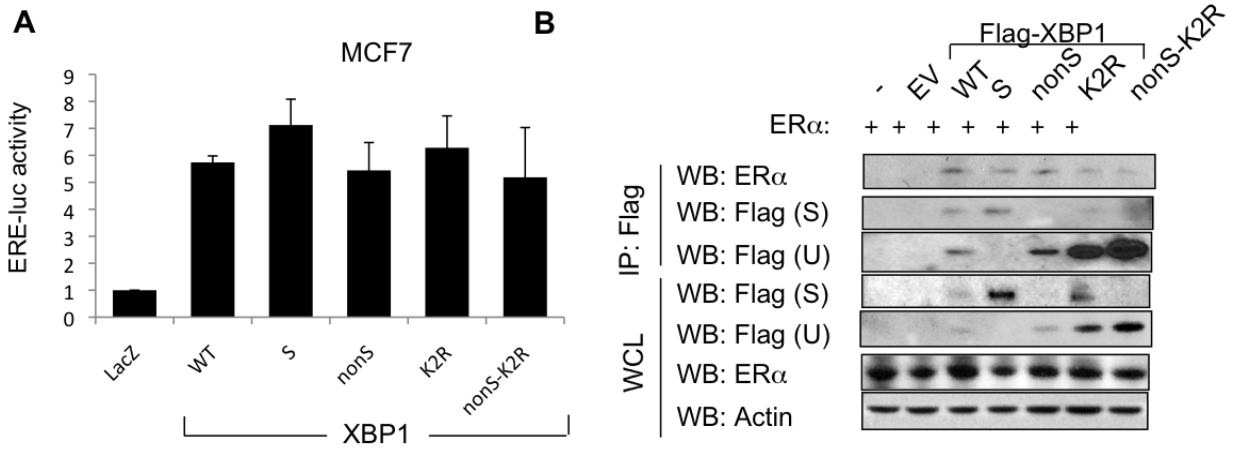

Figure 1. XBP1 regulates ER alpha signaling. (A) XBP1 constructs were transfected into MCF7 cells together with pGL3-Basic or pGL3-ERE-luc luciferase constructs.

To further confirm our finding, we have used MD-MBA-231 cells, which are ER negative and lacks ER alpha signaling. Similarly, we transfected these cells with XBP1 constructs and measured NFkappaB signaling via NFkappaB-luc**(Figure 2)**. We have found that elevated NFkappaB was only observed with XBP1(S)-overexpressed MD-MBA-231 cells, but not the XBP1(U)-overexpressed cells. Furthermore, when we performed the same experiment with ER-alpha overexpressed MD-MBA-231 cells, we observed activation of NFkappaB signaling in both XBP1(S) and XBP1(U) overexpressed cells. These data together suggest that ER-alpha signaling is required for the XBP1(U)-mediated NFkappaB activation.

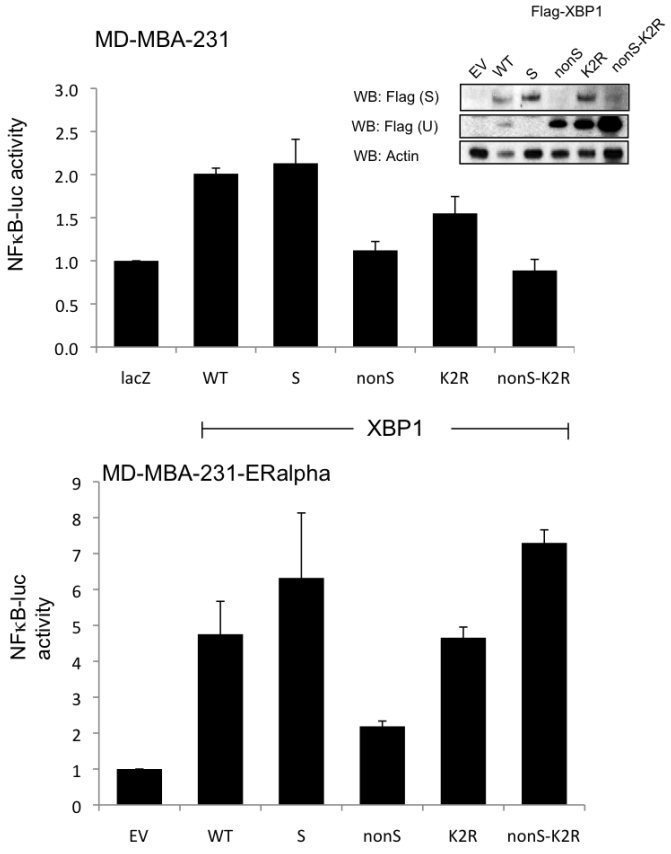

Figure 2. The effects of XBP1 on NFkappaB signaling require ERalpha signaling in MD-MBA-231 cells. Different XBP1 constructs were transfected into MD-MBA-231 or MD-MBA-231-ERalpha cells, together with pGL3-Basic or pGL3-NFkappaB-luc luciferase constructs. NFkappaB signaling activity was measured by NFkappaB-luciferase activity twenty-four hours after transfection.

We have further generated MCF7 cells that stably overexpress XBP1(S) and XBP1(U) cell lines through lentiviral infection**(Figure 3)**. Consistent with our previous finding, we have found that MCF7-XBP1(S) cells is more resistant to both Tamoxifen and ICI than lacZ overexpressing control cells. However, MCF7-XBP1(U) cells, which also had enhanced NFkappaB signaling, only displayed moderate resistance Tamoxifen, and similar sensitivity to ICI comparing to the control cells. These data suggest that the XBP1(S) mediated antiestrogen resistance relies partly on NFkappaB signaling, and other signaling pathways might also be involved for antiestrogen resistance.

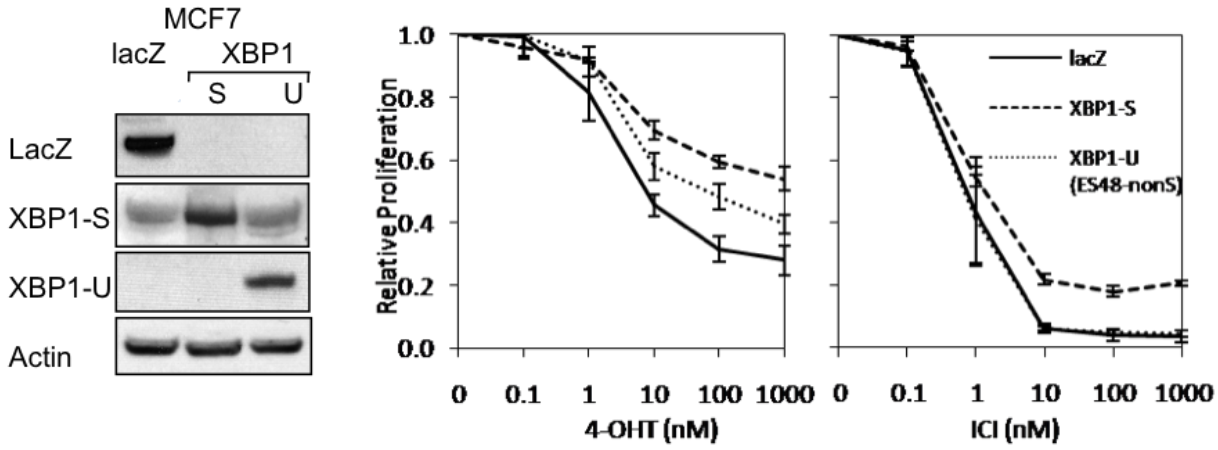

Figure 3. MCF7 cells that stably overexpress XBP1(S) and XBP1(U) cells were generated. XBP1(S) overexpressed cells were more resistant to Tamoxifen and ICI, whereas XBP1(U) overexpressed cells showed only moderate resistance to Tamoxifen and almost no resistance to ICI.

Aim 2: Determine the role of XBP1 in breast cancer antiestrogen resistance *in vivo*.

In the past year, we have obtained the animal protocol approval from DOD-IACUC, and conducted a pilot study with 25 nude mice. We have obtained ovaritarized female nude mice to remove the intrinsic estrogen cycle that might interfere with our study. We then implant an estrogen pellet under skin of each animal to provide sufficient and stable estrogen. We then injected MCF7 cells that overexpress lacZ, XBP1-S and XBP1-U into the mammary fat pad of these mice. Due to the fact that we observe slight but not significant difference in cell growth in vitro, we decided to inject all three different cell lines into the same animal whenever possible. This strategy is aim to minimize the variance between individual mice.

All cell injections were successful without difference in take rate among three cell types (allcells displayed a 100% take rate). However, different celltypes displayed different growth rate drastically**(Figure 4)**. The lacZ-overexpressed tumors grow the slowest among the three. As expected, XBP1-S overexpressed tumors grow faster than the lacZ cells. However, surprisingly, the tumor growth rate of XBP1-U overexpressed tumors was dramatically enhanced. These

results suggest that an unknown mechanism might be involved in promoting the growth of XBP1(U) overexpressed tumors. In order to confirmed the overexpression of XBP1(S) and XBP1(U), we have collected tumor tissue samples from animals. Our western blot analysis demonstrated that our strategy is successful, and XBP1 are stably overexpressed during our *in vivo* experiments.

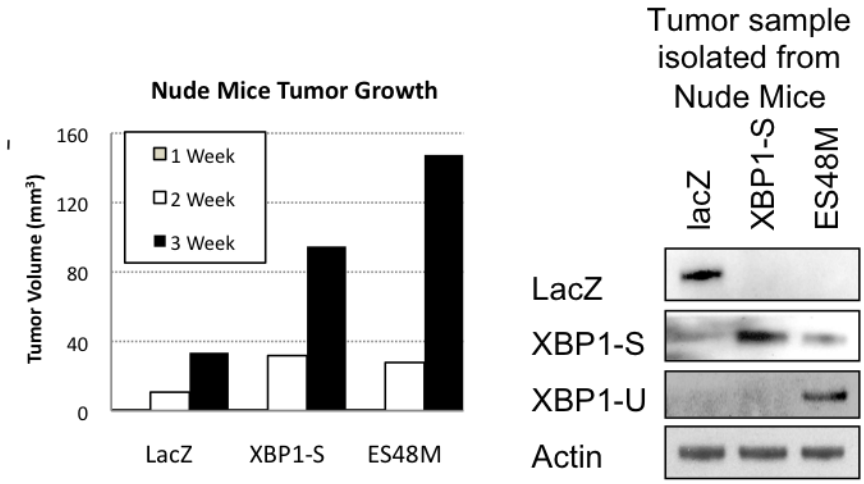

Figure 4. XBP1(S) and XBP1(U) overexpressed tumors grow much faster than LacZ control tumors. The right panel was samples collected from harvest tumor tissues to confirm the overexpression of XBP1 and LacZ as indicated.

Due to the fast growth of the XBP1-U tumor, we often had to sacrifice the animal before the lacZ and XBP1-S tumor reached the needed size to start drug treatment. Even thoughwe had to terminate the pilot experiment earlier than initially planned, the discovery found in this pilot study was valuable. The results from this *in vivo* experiment strongly contrasted our observation *in vitro*, underscored the importance of *in vivo* animal experiments. In addition, we obtained further insights about these cells from the tissue samples we harvestedfrom the animals in this pilot study. When we sacrifice these animals, we often observe that XBP1-S and XBP1-U tumors are better vascularized than the lacZ tumors, suggesting a role of XBP1 in regulating angiogenesis. We have stained the tissue samples with proliferation marker Ki67 and apoptosis marker TUNEL**(Figure 5)**. Our data suggest that the XBP1-S and XBP1-U cells displayed enhanced proliferation and apoptosis, which is consistent with the enhanced tumor growth observed in these tumors. Furthermore, we have also stained the tissue samples with angiogenesis markers CD31. Similar to the enhanced vascularization that we have observed during tissue collection, we observed enhanced and clustered CD31 staining in XBP1(S) and XBP1(U) overexpressed tissues. However, due to different tumor sizes between XBP1 and LacZ tumor samples. Further investigation is needed to draw conclusions between XBP1 and angiogenesis.

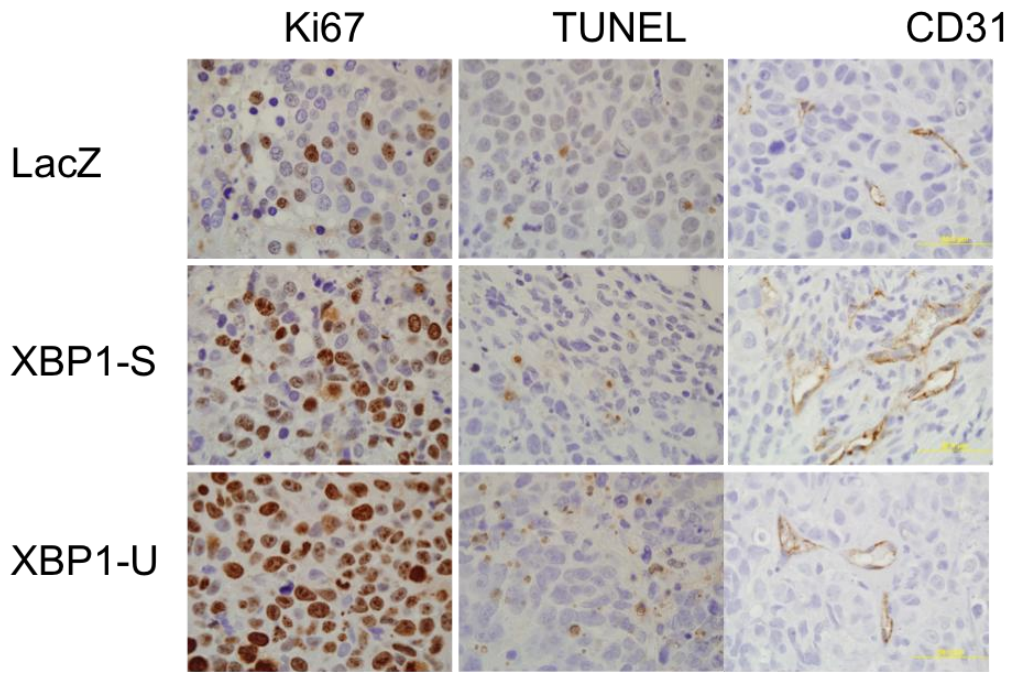

Figure 5. Representative pictures from tumor sample slide stained with proliferation marker Ki67, apoptosis marker TUNEL and angiogeneisis marker CD31.

Based on the findings from our pilot study, we are eager to further examine the effects of XBP1 overexpression on tumor growth. In addition, we are interested in determine the role of NFkappaB signaling in regulating the enhanced tumor growth. We were recently granted renewal of our animal protocol from GUACUC, and my animal protocol is currently under renewal revision by DOD-IACUC. After the approval, we planto initiate our next animal experiments. We will adjust our strategy based on the observation from our pilot study. Instead of inject cell types into the same animal; each animal will be injected with the same cell type. Furthermore, we will start the treatment of animals with Tamoxifen and NFkappaB inhibitor Parthenolide based on the size of the tumor. We also aim to further determine the relationship between XBP1 and angiogensis both *in vivo* and *in vitro* investigation.

III. Key Research Accomplishments

- XBP1(U) and XBP1(S) both bind to and regulates ERalpha signaling
- The up-regulation of NFkappB signaling by XBP1(U) and XBP1(S) requiresERalpha.
- XBP1(U) cells are moderately resistant to Tamoxifen and still sensitive to ICI
- XBP1(S) overexpression promotes tumor growth in xenograft model

- XBP1(U) overexpressed MCF7 cells form tumors even faster than XBP1(S) overexpressed cells in xenograft model
- XBP1(S) and XBP1(U) overexpressed tumors are better vascularized and may contribute to angiogensis.

IV. Reportable Outcomes

Manuscriptsin preparation:

Hu R., Eltayeb A., Tabor K., Shajahan A., Riggins R., Clarke R. NF□ B Signaling up-regulation is required for XBP1 Mediated Antiestrogen Resistance in Breast Cancer Cells. Manuscript in preparation.

Cook KL., Hu R., Jin L., Zwart A., Clarke R., Warri A. Unfolded protein response and autophagy regulate the reversible stage of mammary involution preceding apoptosis.

Manuscripts

Clarke R., Cook KL., Hu R., Facey COB., Tavassoly I., Schwartz J., Baumann WT., Tyson JJ., Xuan J., Wang Y., Warri A., Shajahan AN. 2012 Endoplasmic reticulum stress, the unfolded protein response, autophagy, and the integrated regulation of breast cancer cell fate. Cancer Res. 72: 1321-1331.

Harini SA.,Bouker KB., Cook KL., Facey COB., Hu R., Schwartz J., Shajahan AN., Hilakivi-Clarke L., Clarke R. 2012 Interaction of dietary polyphenols with molecular signaling pathways of antiestrogen resistance: possible role in breast cancer recurrence. Hormones in Normal and Cancerous Breast: Basic and Clinical Applications. In press

V. Conclusions

From the second year of the funded research, we have made several positive findings. First, we determined the role of ER-alpha signaling in regulating NFkappaB signaling by XBP1 in breast cancer. We found that ER-alpha signaling is essential for activating NFkappaB signaling in XBP1(U) overexpressed cells, which we have demonstrated both in ER-alpha positive and negative breast cancer cell lines. Second, we found that XBP1(U) display partial resistance to Tamoxifen and no resistance to ICI, even though it also has enhanced NFkappaB and ER-alpha signaling. Third, we have observed that the growth rate of XBP1 overexpressed tumors is greatly enhanced, especially the XBP1(U) tumors. Finally, our observation suggested a link between XBP1 and angiogenesis, even though further investigation is clearly needed.

The discoveries made from the past two years lead us to additional exciting questions. We have established a link between XBP1 and NFkappaB signaling, possibly through ER-alpha in breast cancer cells. As a survival signaling pathway that has been activated in many cancer types,

NFkappaB signaling is an active target for therapeutics. According to the data I obtained from my study, NFkappaB signaling plays an essentialrole in XBP1-driven antiestrogen resistance in breast cancer. Co-treatments that targeting both signaling pathways for synergistic effects should be examined for antiestrogen resistant breast cancer model.The in vivo xenograft experiment that we plan to undertake will serve to test this hypothesis. In addition, we found unexpected roles of the unsplicedXBP1(U). It not only binds to and activates ER-alpha signaling and thus NFkappaB signaling, it also strongly promotes tumor growth *in vivo*. However, the underlying mechanisms are still unclear, and our preliminary results suggest that angiogenesis might be involved. In future studies, we will further investigate the effects of XBP1 (both U and S) on angiogensis*in vivo* and also *in vitro* investigation.

VI. References

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VII. Appendices

Current *curriculum vitae* (3 pages).

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel on page 1 of the Detailed Cost Estimate form for the initial budget period.

NAME	POSITION TITLE
Rong Hu	Post-doctoral Fellow

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Nanchang University	B.S.	1999-2003	Biotechnology
University of Leicester	M.Sc.	2003-2004	Molecular Genetics
SUNY-Albany		2004-2005	Molecular Biology
Albany Medical college		2005-2008	Cancer Biology
Thomas Jefferson University	Ph.D	2008-2009	Genetics
Georgetown University Medical Center	Post-Doc	2010-Present	Breast Cancer

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List in chronological order the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 4 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INDIVIDUAL.

Research Experiences:

09/2003-08/2004: MSc. In Molecular Genetics, University of Leicester, Leicester, UK
Thesis: Mechanism of Translational Selection of mRNAs during Apoptosis
Mentor: Dr. Martin Bushell

06/2005-11/2009: Ph.D in Genetics, Albany Medical College and Thomas Jefferson University
Thesis: F-box proteins and co-factors of SCF E3 ubiquitin ligases in melanoma.
Mentor: Dr. Andrew Aplin

01/2010-Present: Post-doctoral Fellow, Georgetown University
Research: Role of UPR signaling in endocrine resistance in breast cancer
Mentor: Dr. Robert Clarke

Publications and Manuscript in preparation:

Bhatt KV., **Hu. R.,** Spofford LS., Aplin AE. (2007) Mutant B-RAF signaling and cyclin D1 regulate Cks1/S-phase kinase-associated protein 2-mediated degradation of p27Kip1 in human melanoma cells. Oncogene. 26(7): 1056-1066.

Hu R. and Aplin AE. (2008) Skp2 regulates G2/M progression in a p53-dependent manner. Mol. Biol. Cell. 19(11): 4620-4610.

Hu R. and Aplin AE. (2009) αB-crystallin is mutant B-RAF regulated and contributes to cyclin D1 turnover in melanocytic cells. Pigment Cell & Melanoma Research. 2010 Jan 27. Epub ahead of print.

Clarke R., Cook KL., **Hu R.,** Facey COB., Tavassoly I., Schwartz J., Baumann WT., Tyson JJ., Xuan J., Wang Y., Warri A., Shajahan AN. (2012) Endoplasmic reticulum stress, the unfolded protein response, autophagy, and the integrated regulation of breast cancer cell fate. Cancer Res. 72: 1321-1331.

Harini SA., Bouker KB., Cook KL., Facey COB., **Hu R.,** Schwartz J., Shajahan AN., Hilakivi-Clarke L., Clarke R. 2012 Interaction of dietary polyphenols with molecular signaling pathways of antiestrogen resistance: possible role in breast cancer recurrence. Hormones in Normal and Cancerous Breast: Basic and Clinical Applications. In press

Hu R., Eltayeb A., Tabor K., Shajahan A., Riggins R., Clarke R. NFkappaB Signaling up-regulation is required for XBP1 Mediated Antiestrogen Resistance in Breast Cancer Cells. Manuscript in preparation.

Cook KL., **Hu R.,** Jin L., Zwart A., Clarke R., Warri A. Unfolded protein response and autophagy regulate the reversible stage of mammary involution preceding apoptosis.

Funding:

Pre-doctoral Fellowship, National Cancer Center, 09/2007-09/2009
Skp2 regulation of melanoma cell proliferation: mechanism and role in a skin-like microenvironment

Post-doctoral Fellowship, Department of Defense, 09/2010-09/2013
Role of NFkB signaling in X-box binding protein 1(XBP1)-mediated antiestrogen resistance in breast cancer

Conferences:

Nov. 2007 International Melanoma Congress New York, NY
Poster: p53 and cyclin E1-dependent effects of Skp2 on melanoma cell cycle

Apr. 2008 AACR Annual Meeting San Diego, CA
Poster: Skp2 regulates G2/M progression in a p53-dependent manner

Sep. 2009 PanAmerican Society for Pigment Cell Research Annual Meeting Memphis, TN
Poster: F-box protein co-factor Cks1 and αB-crystallin: B-RAF regulation and roles in melanoma cell cycle progression

Apr. 2011 AACR Annual Meeting Orlando, FL
Poster: XBP1 regulates NFkB signaling in Antiestrogen resistant breast cancer cells

Apr. 2011 Experimental Biology Annual Meeting Washington, DC

Poster: IRF1 promotes antiestrogen sensitivity by regulating Bik expression in breast cancer cells

Aug. 2011 Era of Hope Breast Cancer Meeting Orlando, FL
Poster: NFkB Signaling is required for XBP1-mediated antiestrogen resistance in breast cancer

Awards and Honors:

Distinguished Student Award, Nanchang University, 2002, 2003

Student Scholarship, Nanchang University, 2000, 2001, 2002, 2003

Distinguished Student, University of Leicester, 2004

Richard A. Miller Alumni Prize, Albany Medical College, 2008

Dean's Excellence in Extramural Research Activities, Albany Medical College, 2008

Travel Award for 15th Annual Meeting of the PanAmerican Society for Pigment Cell Research, 2009

1st place in Poster Award for 15th Annual Meeting of the PanAmerican Society for Pigment Cell Research, 2009

Mentoring:

Irene Thung: Irene was a medical student at Georgetown University who performed her research intern in Dr. Clarke's laboratory. In summer 2010, I worked with Irene toward the completion of her research project, which focuses on understanding the effects of Akt/mTOR dual inhibitors in antiestrogen-resistant breast cancer.

Ahreej Eltayeb: Ahreej was a graduate student at George Washington University who performed her research in Dr. Clarke's laboratory. From November 2010, I worked with Ahreej toward investigating the role of XBP1 splicing in antiestrogen resistance in breast cancer. I assisted Ahreej in crafting her research plan for the supplemental RO1 award that she received. Ahreej is now working as a full-time technician in Dr. Clarke's lab.

Katie Tabor: Katie is a medical student at Georgetown University working in Dr. Clarke's laboratory for her independent study project. From summer 2011, I worked with Katie on her project on understanding the role of unspliced form of XBP1 (XBP1-U) in apoptosis and autophagy in breast cancer.

Amanda Rosen: Amanda is a senior student at Georgetown University working towards her Bachelors' degree. She is working in Dr. Clarke's laboratory for undergraduate thesis. From summer 2012, I worked with Amanda on her project on understanding the mechanism of up-regulated XBP1 in LCC9 antiestrogen resistant breast cancer cells.